IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY *et al.*

Serial No.: 09/724,726 Group Art Unit: 1638

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For: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Steven F. Fabijanski, declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000.

2) I received a Bachelor's degree in Biology from the University of Miami (Florida) in 1977. I received a Ph.D. degree in Cellular and Molecular Biology from the University of Southern California in 1981. I have held post-doctoral positions at the University of Ottawa in Ottawa, Ontario, Canada and the University of Southern California in Los Angeles, California from 1982 to 1985. From 1986 to 1991, I held the position of Research Director at Paladin Hybrids, Inc.

3) I have over 20 years of experience in the areas of plant molecular biology, plant gene expression, plant tissue and cell culture and development of techniques to produce genetically modified plants and plant artificial chromosomes. I have authored or co-authored over 20 publications and I am an inventor of 15 US and foreign patents.

4) I am currently Research Director at Agrisoma Biosciences Inc., located in Saskatoon, Saskatchewan, Canada. Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9, is an owner of Agrisoma Biosciences, Inc. I have held this position since 2001. I am also President of FAAR Biotechnology Group Inc., located at Suite 323, 5929L Jeanne D'arc Boulevard, Orleans, Ontario, Canada K1C 7K2. I have held this position since 1992.

5) In my capacity as researcher and Director of Research, myself, persons under my direction and other research groups: the Scottish Crop Research Institute in Scotland; the Danforth Plant Science Center in St. Louis, Missouri; the Hungarian Biological Research Center in Hungary; Chromos Molecular Systems, Inc., Burnaby, B.C., Canada; and Applicant's research group at the Plant Biotechnology Institute in Saskatoon, Saskatchewan, Canada; have introduced mammalian satellite artificial chromosomes into plant protoplasts, generated plant satellite artificial chromosomes in plant protoplasts and generated transgenic plants containing plant satellite artificial chromosomes. The results of some of these studies have been presented in three previous Declarations under my signature that were submitted to the U.S. Patent and Trademark Office on July 16, 2003, April 22, 2004 and January 6, 2005 (referred to herein as Fabijanski Declarations 1, 2 and 3, respectively). Accordingly, this Declaration is the fourth to present results of these studies (i.e., Fabijanski Declaration 4).

6) Upon further review of my first Declaration under 37 C.F.R. §1.132 (Fabijanski Declaration 1) that was submitted to the U.S. Patent and Trademark Office on July 16, 2003, an inadvertent error was discovered. The error is in the designation of a cell line referred to in the declaration. Specifically, in that declaration (see p. 2 of Fabijanski Declaration 1), the cells containing a satellite artificial chromosome from which microcells were prepared was pAgII/B19-18, not the EC3/7C5 cell line referred to in the declaration. The pAgII/B19-18 cell line is, however, correctly described in Fabijanski Declaration 1 as a murine cell line containing a satellite artificial chromosome. The satellite artificial chromosome was generated by transfecting mouse LMtk$^-$ cells with DNA encoding a selectable marker (puromycin-resistance), DNA containing an attP recombination site and mouse rDNA. The vector pAgIIa (described herein and in Fabijanski Declarations 2 and 3) was subsequently incorporated, in its entirety, into the satellite artificial chromosome via lambda integrase-mediated site-

specific recombination. Although the resulting satellite artificial chromosome contained all the elements of vector pAglla, it had a mammalian (mouse) centromere and mammalian heterochromatin. Microcells were prepared from the B19-18 cells containing the resulting satellite artificial chromosome that had incorporated pAglla into it. The microcells were fused with tobacco BY-2 protoplasts as described in Fabijanski Declaration 1.

7) Using methods and materials such as those described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have introduced heterologous DNA into plant protoplasts, analyzed cells of calli resulting from the transfected protoplasts and identified a plant satellite artificial chromosome contained therein. The results of this work demonstrate that a plant satellite artificial chromosome generated following the teachings in the above-captioned application possesses the structural, physical and functional characteristics of a plant satellite artificial chromosome described in the above-captioned application.

I. Materials and Methods

Generation of Plant Satellite Artificial Chromosomes

1. Construction of heterologous DNAs

Vector pAglla, containing two plant selectable markers, one bacterial selectable marker (*i.e.*, DNA encoding kanamycin resistance), an attB recombination site adjacent to a promoterless zeomycin resistance-encoding DNA, and a sequence with homology to pericentric DNA was constructed using standard techniques of molecular biology. One plant selectable marker that was incorporated into the vector was a hygromycin phosphotransferase (HPT)-encoding sequence under the control of the 35S promoter (see, *e.g.*, Blochinger and Diggelmann, *Mol. Cell. Bio. 4*:2929-2931). A 334 bp sequence with homology to tobacco pericentric sequences (Genbank Accession no. Y08422 submitted 1996; see also Genbank Accession nos. X76056 and D76443,

submitted 1993 and 1995, respectively) was constructed, containing the central AT-rich region of a tobacco rDNA intergenic spacer capable of amplification (Borisjuk *et al.* (1997) *Plant Mol. Bio. 35*:655-660), and incorporated into the vector. Vector pAgIIa also contains a visible marker, constructed by placing DNA encoding β-glucuronidase (GUS) under the control of the nos promoter (Novel and Novel (1973) *Mol. Gen. Genet. 120*:319-335; Jefferson *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*:8447-8451; U.S. Patent No. 5,268,463; commercially available from Clontech Laboratories, Palo Alto, CA), and a detection marker containing a 234-bp mouse major satellite DNA sequence derived from pSAT-1 (Wong and Rattner (1988) *Nuc. Acids Res. 16*:11645-11661). The vector also contains a second plant selectable marker constructed from a phosphinothricin acetyl transferase (PAT) gene under the control of the 35S promoter (see, *e.g.*, White *et al.* (1990) *Nuc. Acids Res. 18*:1062; Spencer *et al.* (1990) *Theor. Appl. Genet. 79*:625-631; Vickers *et al.* (1996) *Plant Mol. Biol. Reporter 14*:363-368; and Thompson *et al.* (1987) *EMBO J. 6*:2519-2523).

A targeting DNA was constructed with homology to pericentric DNA sequences. The targeting DNA contains a 1.7 kb portion of the 26S rDNA coding region from the *Arabidopsis* rDNA repeat unit (Genbank Accession no. X52320). The targeting DNA was cloned into vector pBluescript (Stratagene, La Jolla, CA).

2. Introduction of DNAs into plant cells and selection

Vector DNA and targeting DNA were introduced into tobacco cells using PEG-mediated transfection. Briefly, tobacco protoplasts were isolated from established sterile tobacco plant cultures by immersion of sterile tissue in enzyme solution containing 1.2% Cellulase 'Onozuka' R-10 and 0.4% Macerozyme R-10. The protoplasts were purified by pouring through a 100 μm nylon mesh sieve, overlaid with washing solution and centrifuged at 80 x g for 10 minutes. Protoplasts were then resuspended at a density of 1 x 10^6 protoplasts/ml and stored at 4°C for 1 to 2 hours prior to DNA uptake.

The vector and targeting DNAs were sterilized with chloroform and 70% ethanol before use. A protoplast suspension was mixed with vector and targeting DNA at a ratio of 1:10 followed immediately by slow addition of a polyethylene glycol (PEG) solution. To examine the effect of using a 10-fold excess of targeting DNA relative to vector (pAgIIa) DNA, in some transfections, salmon sperm DNA or calf thymus DNA was added instead of targeting DNA. The mixtures were incubated at 22°C for 10-15 minutes with gentle shaking. The protoplasts were resuspended and cultured at 22°C in the dark. When microcalli developed, the protoplasts were embedded in 0.6% agarose. Selection on protoplast cultures was carried out by adding hygromycin to the medium at a final concentration of 20 mg/l, 14 to 21 days after transfection. Calli that grew on selection were cultured under selective conditions for a period of 3-6 months with frequent subculturing. Standard molecular biology techniques were used to verify the presence of the vector DNA.

3. Identification of amplified DNAs

GUS-expressing calli that were produced using vector DNA and either targeting DNA or other (i.e., salmon sperm DNA or calf thymus) DNA were subjected to two-color fluorescent in situ hybridization (FISH) using two probes. The first probe was tagged with rhodamine (red fluorescence) and recognized pericentric DNA (18S rDNA) sequences endogenous to tobacco cells. The second probe recognized the detection marker (mouse major satellite sequence) in the vector pAgIIa used for transfection and was visualized with a fluorescein isothiocyanate (FITC) tag (blue/green fluorescence).

To obtain spreads of metaphase chromosomes, cells were subjected to either a single blocking protocol (colchicine treatment) or double blocking protocol (for example, treating plant cells with 5 mg/l aphidocolin for 24 hours and then 1.54 mg/ml Propyzamide for 4 hours). Blocked cells were recovered and chromosome spreads prepared and subjected to two-color FISH. Red and blue/green fluorescence was monitored to identify amplification. In general, 8-

10 chromosome spreads were screened per sample. Further fluorescent image analysis was performed in a subset of the samples to overlay the probe signals and further detail chromosome structure.

II. Results

The results demonstrate that plant satellite artificial chromosomes generated in plant cells have the characteristic structural features (*e.g.*, arms and a centromeric region) and functional features (replication and maintenance in plant cells) of a chromosome and can be identified and distinguished on the basis of the predominance of heterochromatin in the chromosome as well as size differences following amplification.

Following introduction of the heterologous DNAs into tobacco cells, cells were selected on hygromycin. More than 400 calli were obtained. A portion of the calli were analyzed for expression of the GUS reporter gene. A total of 31 independent GUS-expressing calli obtained using the vector and either the targeting DNA or other (*i.e.*, salmon sperm DNA or calf thymus) DNAs were selected for further analysis.

The calli were subjected to two-color FISH using the probes for endogenous pericentric (tobacco 18S rDNA) sequences and for the detection marker (mouse major satellite DNA sequence) in the vector. The pericentric rDNA loci on tobacco chromosomes stained red, and regions of the chromosomes where vector DNA inserted stained blue/green.

Chromosome spreads from 24 calli produced after transfection of tobacco protoplasts with vector and targeting DNA were analyzed for staining with the marker-specific probe. Blue/green signal intensity ranging from low to medium and high was observed. The presence of a medium-to-high blue-green signal revealed loci at which amplification of the vector DNA had occurred. In contrast, a low blue/green signal was indicative of insertion of vector DNA without large-scale amplification of the vector sequence. Of the 24 calli analyzed, 5 were categorized as having a medium level of blue/green signal, 1

as having a high signal and 1 as having a medium-high signal. The remaining 17 samples yielded a low blue/green signal. The variation in signal intensity observed in this small population of transfectant cell lines is related to the extent of amplification associated with homologous recombination into the pericentric DNA as well as the random integration that also occurs and does not result in large-scale amplification of integrated DNA.

The FISH analyses reveal that at least 7 integration events were a specific colocalization of exogenous DNA with pericentric DNA as a result of homologous recombination. The remaining 17 calli analyzed did not show specific colocalization of exogenous DNA to percentric DNA and are presumably the result of random integration that invariably takes place upon introduction of foreign DNA into cells. Thus, in 7 of the 24 analyzed calli produced by transfection of protoplasts with vector and an excess of targeting DNA homologous to pericentric DNA sequences, large-scale amplification of the vector sequences was observed at the chromosome level. Using an excess of targeting DNA produced integration events with large-scale amplification of DNA at a rate of close to 30% of the events analyzed, indicating a very efficient process.

The chromosome spreads from eight calli resulting from transfection of protoplasts with vector DNA and salmon sperm or calf thymus DNA yielded low levels of blue/green signal. Thus, large-scale amplification was not observed in chromosome spreads of these eight calli produced by transfection of protoplasts with vector and either salmon sperm or calf thymus DNAs. As described in the above-captioned application, large-scale amplification can be induced by insertion of heterologous DNA into the pericentric DNA; thus, to enhance the occurrence of such integration, the heterologous DNA can contain sequence homologous to pericentric DNA for "targeted" homologous recombination. In accordance with the teachings of the application, these results illustrate that reducing the excess of targeting DNA (*i.e.*, DNA with homology to pericentric

DNA) similarly reduces the efficiency of integration of the exogenous DNA into the pericentric heterochromatin and thereby reduces large-scale amplificiation of the DNA.

The vector pAgIIa contains a small 334 bp region homolgous to a portion of the tobacco rDNA repeat structure. As is generally known in the art however, a small region of homology to a specific region of a chromosome is usually not sufficient to provide an efficient mechanism for homologous recombination to occur with high frequency. Comparison of the results of the transfections using pAgIIa and an excess of DNA containing a larger region (*i.e.*, 1.7 kb) of homology to pericentric DNA with the results of transfections using pAgIIa without an excess of such DNA (*i.e.*, using salmon sperm or calf thymus DNA) demonstrates that an excess of a larger sequence with homology to pericentric DNA provides for more efficient homologous recombination than a lesser amount of a smaller homologous sequence. It is further noted that chromosome spreads from only eight calli produced by transfection of protoplasts with vector pAgIIa and either salmon sperm or calf thymus DNA were analyzed by FISH for level of blue/green staining. Because efficiency of homologous recombination of the pAgIIa 334-bp sequence with homology to pericentric DNA is less than that of the targeting DNA 1.7-kb sequence with homology to pericentric DNA, it would be necessary to analyze a greater number of calli produced with the vector and the other DNA in order to observe a large-scale amplification event induced by integration of the vector into the chromosomal pericentric DNA.

Each of the chromosome spreads were also analyzed for staining with the probe specific for pericentric (tobacco 18S rDNA) sequences. Because the probe recognizes the 18S rDNA present in any of the tobacco chromosomes, red signals were observed in each of the chromosome spreads.

A comparison of blue/green-staining (to detect vector DNA) and red-staining (to detect 18S rDNA) of the same chromosome spread from one of the

callus lines yielding a high level of blue/green staining revealed overlap of the blue/green and red signals (see Figure 1). These results provide evidence that homologous recombination between the exogenous DNAs and rDNA of the tobacco chromosomes had occurred. Areas where significant levels of both blue/green and red signals were observed demonstrate large-scale amplification of the pericentric DNA. As shown in Figure 1, red-staining of the chromosomes revealed eight visible rDNA loci. Comparison of the red and blue/green signals demonstrates that the vector was inserted and amplified in three of these eight loci.

For example, for one of the rDNA loci (as indicated by red-staining), a comparison of the blue/green and red staining revealed a repeated tandem duplication of vector and pericentric DNA signals which could be identified as a sausage amplification of a formerly dicentric chromosome based on the description of satellite artificial chromosome generation in the above-captioned application. This structure (marked in Figures 2A-C by a white arrow labeled "SAR") appears to contain the amplified heterologous and pericentric DNA as a part of a larger chromosome and thus is referred to as a formerly dicentric chromosome or sausage chromosome from which the amplified segment has not yet broken off as a fragment. Also shown in the upper left corner of Figures 2B and 2C is another structure that stained with blue/green and red stain indicating another pericentric region of a chromosome into which the vector had integrated. The chromosome appears to be an acrocentric chromosome containing rDNA on the short arm which has undergone amplification.

Figures 1 and 2 also show that one of the areas of overlap of the red and blue/green signals is a small, independent breakage product (see circled area of the panels in Figure 1) that was identified as a plant satellite artificial chromosome upon further detailed analysis described herein. The comparison of Figure 2, panels A-C (DAPI-, blue/green- and red-stained chromosomes, respectively), clearly depicts this independent artificial chromosome containing

amplified pericentric and vector DNA (see entity marked with a yellow arrow labeled "SATAC"). It is noted that this plant satellite artificial chromosome was generated through an initial transfection of tobacco protoplasts with heterologous DNA containing *Arabidopsis* 26S rDNA whereas the rhodamine-labeled probe for pericentric DNA used in the FISH analysis was specific for tobacco 18S rDNA. Thus, the rDNA signal obtained in this analysis represents newly amplified rDNA, and the signal cannot be attributed to the heterologous DNA.

In more detailed studies of the plant satellite artificial chromosome, image analysis was used to overlay the pericentric rDNA and vector DNA signals. In high-resolution images, the presence of both amplified vector (blue/green signal) and pericentric heterochromatic DNA (red signal) was observed. This is shown in Figure 3A and 3B which provides overlay images from two independent analyses of chromosome spreads of the plant satellite artificial chromosome. These images of the metaphase plant satellite artificial chromosome clearly demonstrate the presence of small chromosome arms and a constriction representing the centromere region. The callus cell line containing the plant satellite artificial chromosome was stably maintained in culture for over six months and repeated analyses of cells during this period revealed the continued presence of the plant satellite artificial chromosome. As described in Fabijanski Declaration 3 (submitted on January 6, 2005), this plant satellite artificial chromosome was also used to generate transgenic plants which also stably maintained the artificial chromosome for over six months.

III. Conclusion

The results of the experiments provided herein demonstrate that by following the teachings of the specification and employing standard methods as described herein, a plant satellite artificial chromosome can be generated and selected within a cell. Accordingly, the description of a plant satellite artificial chromosome provided in the above-captioned application closely correlates with

an actual physical embodiment of the artificial chromosome such that I and other scientists involved in the work were readily able to identify a plant satellite artificial chromosome and distinguish it in a background of plant chromosomes based on the description in the application.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Steven F. Fabijanski

Date: NOV 8 2005